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Targeted Antimicrobial Therapy Against *Streptococcus mutans* Establishes Protective Non-cariogenic Oral Biofilms and Reduces Subsequent Infection

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Abstract

Aim Dental biofilms are complex communities composed largely of harmless bacteria. Certain pathogenic species including *Streptococcus mutans* (*S. mutans*) can become predominant when host factors such as dietary sucrose intake imbalance the biofilm ecology. Current approaches to control *S. mutans* infection are not pathogen-specific and eliminate the entire oral community along with any protective benefits provided. Here, we tested the hypothesis that removal of *S. mutans* from the oral community through targeted antimicrobial therapy achieves protection against subsequent *S. mutans* colonization.

Methodology Controlled amounts of *S. mutans* were mixed with *S. mutans*-free saliva, grown into biofilms and visualized by antibody staining and cfu quantization. Two specifically-targeted antimicrobial peptides (STAMPs) against *S. mutans* were tested for their ability to reduce *S. mutans* biofilm incorporation upon treatment of the inocula. The resulting biofilms were also evaluated for their ability

to resist subsequent exogenous *S. mutans* colonization.

Results *S. mutans* colonization was considerably reduced (9 ± 0.4 fold reduction, $P=0.01$) when the surface was preoccupied with saliva-derived biofilms. Furthermore, treatment with *S. mutans*-specific STAMPs yielded *S. mutans*-deficient biofilms with significant protection against further *S. mutans* colonization (5 minutes treatment: 38 ± 13 fold reduction $P=0.01$; 16 hours treatment: 96 ± 28 fold reduction $P=0.07$).

Conclusion *S. mutans* infection is reduced by the presence of existing biofilms. Thus maintaining a healthy or “normal” biofilm through targeted antimicrobial therapy (such as the STAMPs) could represent an effective strategy for the treatment and prevention of *S. mutans* colonization in the oral cavity and caries progression.

Keywords targeted antimicrobial therapy, antimicrobial peptide, biofilm, *Streptococcus mutans*, protective colonization, caries

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Introduction

Dental caries (tooth decay) is one of the most prevalent and costly diseases in the United States and throughout the world (Evans and Kleinman,

2000). Although the ultimate manifestation of the disease is the dissolution of tooth structure, the biological nature of the disease is a microbial infection caused primarily by the cariogenic bacterium *Streptococcus mutans* (*S. mutans*) (Clarke,

1924; Loesche *et al.*, 1975; Loesche, 1986; Tanzer *et al.*, 2001). Most current dental therapies are focused on eradicating the entire dental plaque *via* mechanical removal or broad-spectrum antimicrobial treatments. However, the improved understanding of oral microbial ecology, especially the importance of the balance between oral pathogens and commensal residents, has prompted interest in novel approaches focused on selective pathogen inhibition and modulation of the microbial composition of dental plaque to control pathogenesis.

Most of these new approaches aim to achieve the eradication of *S. mutans* by targeting its virulence factors, such as the colonization of the tooth surface *via* both sucrose dependent and independent adhesion mechanisms (Koga *et al.*, 2002; Smith, 2002), cell-cell signaling (Lonn-Stensrud *et al.*, 2007), or acid production. Numerous approaches include the development of anti-carries vaccines against either cell surface antigens (Abiko, 2000) or a glucosyltransferase enzyme that is responsible for glucan production (Xu *et al.*, 2007). In an effort to reduce acid production of naturally occurring *S. mutans*, replacement therapy using a genetically engineered *S. mutans* that is able to outcompete indigenous strains while producing significantly less acid has been attempted (Hillman *et al.*, 2000). Several recent studies have also focused on understanding the interactions between *S. mutans* and other neighboring streptococci in the dental plaque and their impact on *S. mutans* pathogenesis. For example, *S. sanguinis* and *S. oligofermentans* have been observed to inhibit *S. mutans* growth through hydrogen peroxide production (Kreth *et al.*, 2005; Tong *et al.*, 2007; Kreth *et al.*, 2009).

Our research group has initiated a targeted approach to control oral microbial pathogenesis *via* a new class of antimicrobials, called specifically-targeted antimicrobial peptides (STAMPs) (Eckert *et al.*, 2006). A typical STAMP molecule consists of two functionally independent moieties conjoined in a linear peptide sequence: a non-specific antimicrobial peptide serves as the killing moiety while a species-specific binding peptide comprises the targeting moiety that provides specific binding to a selected pathogen and facilitates the targeted delivery of the attached antimicrobial peptide. STAMPs have shown effective elimination

of *S. mutans* from a mixed-species environment without affecting closely related non-cariogenic oral streptococci (Eckert *et al.*, 2006). Based on these features, STAMPs have a strong potential for development into novel therapeutics that may selectively eliminate pathogens while preserving the benefits associated with a healthy normal flora.

In the present study, we used a controlled *in vitro* approach to examine the effect of normal flora on *S. mutans* colonization. In particular, we tested the hypothesis that *S. mutans*-specific STAMPs can protect against *S. mutans* infection by maintaining biofilms composed of benign residential species.

Materials and Methods

Strains and growth conditions

S. mutans strains UA140 (Qi *et al.*, 2001) and JM11 (UA140::*pldh-luc*, *Spc*^r) (Merritt *et al.*, 2005) were grown anaerobically (N₂ 85%, H₂ 10%, and CO₂ 5%) in Todd-Hewitt (TH) medium (Difco) or on TH agar plates at 37°C. JM11 was grown in TH supplemented with 800 µg·mL⁻¹ spectinomycin (Sigma, St Louis, USA). For growth of saliva-derived biofilms, TH medium was supplemented with 1% mannose, 1% sucrose, and 1% dextrose. All biofilms were grown anaerobically at 37°C.

Peptide synthesis and purification

Two STAMPs were used in this study: C16G2 (Eckert *et al.*, 2006) and 2_1G2, a derivative in which the *S. mutans*-specific CSPC16 targeting domain was replaced with the newly identified *S. mutans*-specific targeting peptide 2_1 (FIKHFIHFRF). Peptides were synthesized using Fmoc (9-fluorenylmethoxy carbonyl) solid-phase chemistry (431A peptide synthesizer; Applied Biosciences, Foster City, USA) as previously described (Eckert *et al.*, 2006) and purified to >95% by high-performance liquid chromatography (Waters, Milford, USA), as described previously (He *et al.*, 2009). Peptide mass was determined by electrospray ionization (ESI) mass spectrometry (data not shown, 3100 Mass Detector, Waters, Milford, USA).

Screening of saliva samples using confocal laser scanning fluorescence microscopy (CLSM)

An anti-*S. mutans* monoclonal antibody was employed for detecting *S. mutans* colonization within saliva-derived biofilms, as described previously (Gu et al., 2002; Gu et al., 2005). Since this approach cannot distinguish endogenous and exogenously added *S. mutans*, individuals were screened for saliva that was naturally free of *S. mutans* to enable better controlled experiments. For screening, biofilms were grown from unstimulated saliva samples collected from 12 subjects 6 hours post-cleaning. Individual saliva samples were diluted 1:4 into TH supplemented with 1% glucose, 1% mannose and 1% sucrose. This suspension was subjected to a low speed centrifugation for 10 minutes at $600 \times g$ to remove eukaryotic cells and large debris and 400 μL of the supernatant was inoculated into each well of a sterile 8-well Lab-TekTM Chambered Coverglass (Nalge Nunc International; Naperville, USA) that had been coated with 50% (filtered microorganism-free) saliva in phosphate buffered saline (PBS, pH 7.0) for pellicle formation. To enhance attachment of salivary bacteria, 2 cycles of “starter” biofilms were allowed to deposit matrix material on the glass surface for 24 hours followed by removal and chamber re-sterilization, prior to seeding of experimental biofilms. Biofilms were incubated for 16 hours prior to staining and microscopic evaluation.

For visualization, biofilms were stained with the cell permeant general nucleic acid stain SYTO59 (Invitrogen, Carlsbad, USA) according to manufacturer's instructions, and *S. mutans* was specifically identified utilizing SWLA1-IgG2a as described previously (Gu et al., 2002; Gu et al., 2005) with the following modifications: the primary antibody was diluted in PBS to $50 \mu\text{g}\cdot\text{mL}^{-1}$; 100 μL of this diluted antibody solution was used for each sample and incubated for 30 minutes. Biofilms were then washed three times with PBS and further incubated with fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG diluted 1:50 in PBS for an additional 30 minutes according to manufacturer's instructions (Sigma, St Louis, USA). Biofilms were examined with a PASCAL 5 confocal laser scanning microscope using LSM 5

PASCAL software (Zeiss, Jena, Germany). The scanning module of the system was mounted onto an inverted microscope (Axiovert 200M) and samples were viewed through a $40 \times$ oil-immersion objective (Plan-Neofluar/1.3). Excitation at 488 nm with an argon laser in combination with a 505–560 nm bandpass emission filter was used for FITC fluorescence imaging. An excitation at 633 nm with a helium-neon laser and a 650 nm longpass emission filter was utilized to reveal SYTO59-stained cells within the samples.

Saliva samples that appeared free of *S. mutans* were further tested for their ability to integrate exogenously added *S. mutans* UA140 upon co-inoculation. *S. mutans* at 1.25×10^6 cells per well was added directly to the processed saliva and biofilms were allowed to form for 16 hours prior to visualization as described above. Samples without detectable *S. mutans* were eliminated due to their potential ability to suppress *S. mutans* growth. Saliva from subjects containing no detectable *S. mutans* that did not inhibit growth of exogenously added *S. mutans* were utilized for further studies.

Confirmation of protective colonization effects

To test protective biofilm effects, processed saliva suspensions were allowed to form biofilms for 16 hours and then replenished with fresh medium containing 1.25×10^6 *S. mutans* cells. After an additional 24 hours growth period, samples were stained and subjected to CLSM analysis as described above or cfu analysis described below.

Evaluation of STAMP ability to inhibit *S. mutans* biofilm incorporation at inoculation

To test the ability of *S. mutans*-specific STAMPs to remove *S. mutans* at inoculation, $100 \mu\text{mol}\cdot\text{L}^{-1}$ of the respective peptide was directly added to the mixture of salivary bacteria and JM11 (1.25×10^6 cfu $\cdot\text{mL}^{-1}$). The STAMPs would then either be left in the inoculum or be removed via an immediate high speed spin ($16\,000 \times g$) for 5 minutes. The latter samples were washed once and resuspended in an equal volume of fresh medium prior to inoculation. 400 μL of the mixtures were inoculated into each well of a pellicle-coated and UV sterilized 48-well cell culture plate. As bacterial

cells attach significantly better to plastic than to glass surfaces, the “conditioning” process was omitted for these experiments. Biofilms were grown anaerobically for 16 hours, and then washed with PBS and subject to viability evaluation via quantitation of viable colony forming units (cfu) as follows: biofilms were mechanically disrupted by vigorous pipetting in 1 mL TH and a series of 1:10 dilutions was plated onto both TH plates and TH plates supplemented with $800 \mu\text{g}\cdot\text{mL}^{-1}$ spectinomycin to assess total cell viability and JM11 viability, respectively.

Evaluation of STAMP-treated *S. mutans*-free biofilms to inhibit *S. mutans* incorporation

An *S. mutans* JM11 and saliva mixture was prepared and treated with the respective STAMPs as described above prior to biofilm formation for 16 hours before and a subsequent challenge with 1.25×10^6 JM11 cells in fresh medium. After an additional 24 hours of anaerobic incubation, *S. mutans* incorporation was determined by biofilm disruption and plating to quantitate cfu (described above).

Results

Establishment of a saliva-derived biofilm system and observation of protective colonization

Biofilms were grown with saliva collected from subjects who are not colonized by detectable levels of *S. mutans* (selection criteria described in *Methods*) (Figure 1A). To evaluate if we could control exogenous *S. mutans* addition, UA140 was inoculated together with the salivary bacteria to form biofilms which were then examined for *S. mutans* incorporation. *S. mutans* became an integral part of the biofilm community (Figure 1B), comprising $2.7\% \pm 1.0\%$ of the total population after 16 hours of biofilm growth (Figure 1D, 0 hour). This finding confirmed that the *in vitro* saliva-derived biofilm model employed in this study enables the controlled addition of *S. mutans* to address the ability of this bacterium to invade preexisting biofilms.

Interestingly, the proportion of *S. mutans* incor-

porated into the biofilm was significantly reduced ($0.3\% \pm 0.1\%$ of the total biofilm population, representing 9 ± 0.4 fold reduction $P=0.01$) when *S. mutans* was added after saliva biofilms were allowed to form for 16 hours. Although biofilms were grown for an extra 24 hours after addition of *S. mutans*, this further growth period did not significantly increase the number of total biofilm cells ($P=0.14$, Figures 1C and 1D). These results indicate that the observed difference reflects a protective colonization effect when a biofilm is already formed prior to addition of *S. mutans*.

Generation of protective *S. mutans*-free biofilms via STAMP treatment

The results in Figure 1 suggested that the ability of *S. mutans* to colonize a surface was reduced in the presence of a preexisting biofilm derived from natural occurring *S. mutans*-free saliva. Based on this finding, we hypothesized that targeted elimination of *S. mutans* could yield a biofilm with similar protection capability. Previous studies have demonstrated that the STAMP C16G2 possessed specific antimicrobial activity against *S. mutans* cells in both planktonic and early stages of mixed species biofilm formation (Eckert *et al.*, 2006). Here, we evaluated if removal of *S. mutans* from the saliva inoculum with C16G2 or the functionally analogous STAMP 2_1G2 leads to the generation of protective biofilms with the ability to prevent secondary surface colonization by this cariogenic species.

Biofilm inocula containing salivary bacteria and the spectinomycin-resistant *S. mutans* derivative JM11 were incubated with the STAMPs for 16 hours. No *S. mutans* cells were detected in the resulting biofilms for both peptide-treated samples, while the untreated control contained $\sim 10^7$ *S. mutans* cells in the biofilm. More importantly, these *in vitro* generated *S. mutans*-free saliva biofilms were able to prevent subsequent infection from exogenous *S. mutans* (Figure 2). A striking difference in the degree of colonization prevention was apparent in 2_1G2 and C16G2-treated samples: a 96 ± 28 fold ($P=0.01$) and 92 ± 14 fold ($P=0.01$) reduction, respectively, compared to *S. mutans* levels detected in the untreated control (Figure 2).

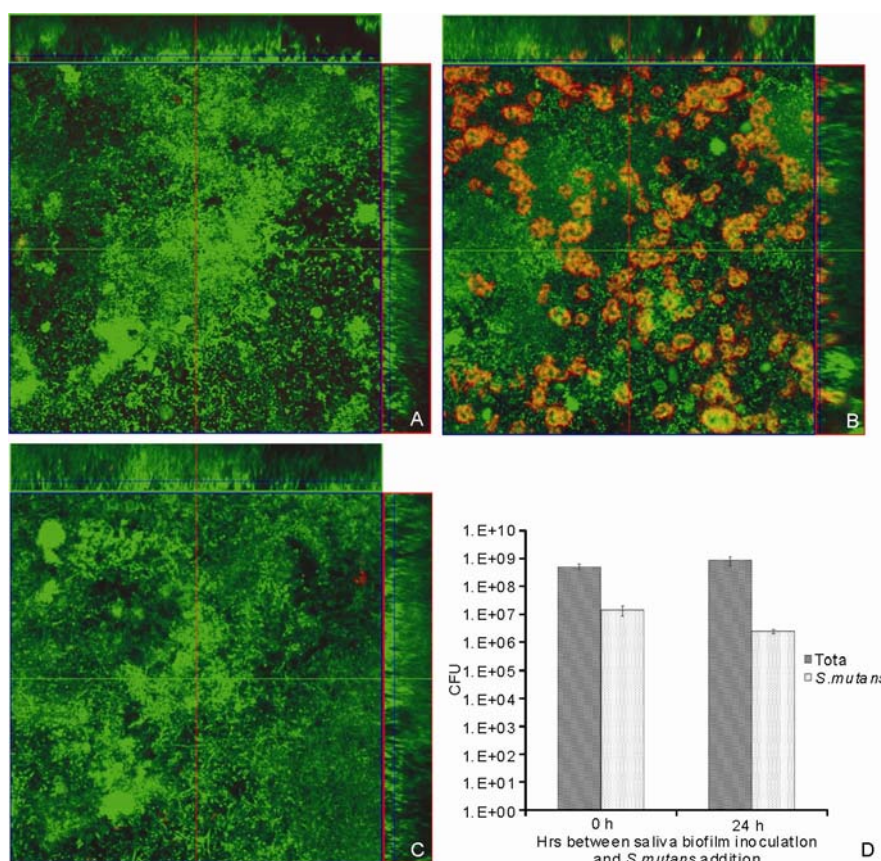


Figure 1 Detection of *S. mutans* in the saliva derived biofilms

Saliva containing no detectable *S. mutans* was chosen for this study. (A)-(C): CLSM imaging. *S. mutans* cells within the biofilm were labeled with anti-*S. mutans* monoclonal antibody and FITC-conjugated secondary antibody (red). SYTO 59 (green) was used to label the total population. (A): 16 hours biofilms derived from saliva. (B): 16 hours biofilms with 1.25×10^6 *S. mutans* UA140 cells spiked at inoculation. (C): 24 hours saliva biofilm was spiked with 1.25×10^6 *S. mutans* cells and allowed to grow for another 16 hours prior to staining. (D): Quantification of *S. mutans* incorporation into saliva biofilm under different growth conditions. 1.25×10^6 *S. mutans* strain JM11 were added to saliva at 0 hour or 24 hours after inoculation (indicated in the figure). cfu of total biofilm cells and *S. mutans* cells are presented, standard deviation of three independent experiments are shown.

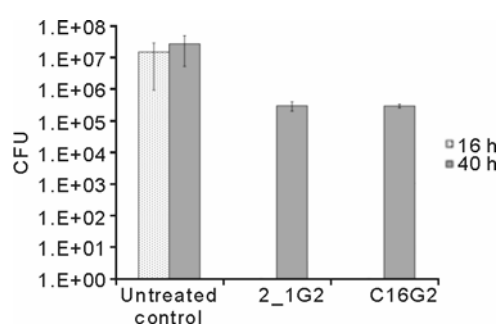


Figure 2 Evaluation of overnight STAMP treatment on *S. mutans* colonization

$100 \mu\text{mol}\cdot\text{L}^{-1}$ STAMPs were added to a saliva/*S. mutans* (1.25×10^6) mixture and incubated anaerobically for 16 hours, *S. mutans* surviving cfu were presented (dotted bars). A duplicate set of samples were washed and replenished with medium containing JM11 cells for additional 24 hours growth. *S. mutans* surviving cfu were presented (grey bars). Standard deviations of three independent experiments are shown.

The overnight treatment with peptides efficiently enabled growth of *S. mutans*-free saliva biofilms despite the presence of significant numbers of *S. mutans* in the inoculum. Based on these encouraging results we further explored the STAMPs' efficacy during shorter exposure times that are most relevant for potential therapeutic applications. As shown in Figure 3, treatment of an *S. mutans* containing saliva inoculum with either STAMP for as little as five minutes prior to biofilm growth resulted in biofilms containing approximately four- (2_1G2) and five- (C16G2) orders of magnitude less *S. mutans* cells (Figure 3). Upon re-infection with *S. mutans*, 38 ± 13 (2_1G2) to 61 ± 13 fold (C16G2) less *S. mutans* cells were detected in treated biofilms compared to the untreated control ($P=0.07$ for both peptides).

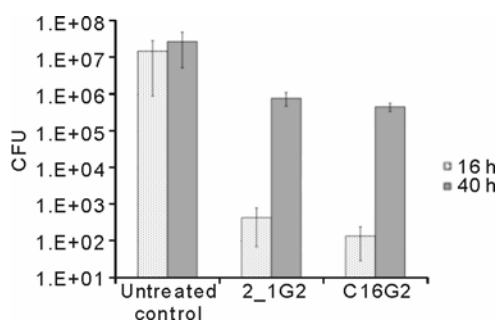


Figure 3 Evaluation of 5 minutes peptide treatment on *S. mutans* colonization

1.25×10⁶ *S. mutans* strain JM11 was added to processed saliva suspensions and treated with 100 μmol·L⁻¹ STAMPs for 5 minutes. Samples were washed and then used to inoculate overnight biofilms. After 16 hours of growth, biofilms were disrupted and plated, *S. mutans* surviving cfu are presented (dotted bars). A duplicate set of samples were washed and replenished with medium containing JM11 cells for additional 24 hours growth. *S. mutans* surviving cfu are presented (grey bars). Standard deviations of three independent experiments are shown.

Discussion

The data presented here support the hypothesis that the cariogenic organism *S. mutans* exhibits greatly reduced colonization in the presence of a preexisting saliva-derived biofilms containing no or a minimal amount of *S. mutans*. These findings provided evidence for the protective effect of a “normal” oral biofilm in concurrence with the proposed “window of infection” identified based on previous clinical studies (Caufield *et al.*, 1993).

According to this report, *S. mutans* colonization mainly occurs between the second to the third year of life. Study participants who were infected during this crucial time period acquired persistent *S. mutans* colonization, while uninfected individuals remained *S. mutans*-free until the eruption of secondary dentition provided new colonization opportunities. It is likely that these uninfected individuals formed a protective biofilm community void of *S. mutans*, similar to the ones we observe in the *in vitro* saliva-derived biofilm model system, which significantly reduced the ability of *S. mutans* to become part of the biofilm community when added later on.

Though the mechanism behind the ability of

biofilms to exclude *S. mutans* remains to be elucidated, recent studies by Kreth *et al.* (Kreth *et al.*, 2005; Kreth *et al.*, 2008) demonstrated that the sequence of inoculation determines whether cariogenic *S. mutans* or the health-associated *S. sanguinis* (Corby *et al.*, 2005; Corby *et al.*, 2007) compete or co-exist with one another. Both species can persist in a biofilm when inoculated at the same time, but given the chance to establish a biofilm first, either species can preclude colonization of the other. In the context of this study, it is possible that the presence of certain species (such as *S. sanguinis*) in the established biofilms could mediate the protective effects against exposure to *S. mutans*. Interestingly, this protective effect was observed even in the presence of 1% sucrose in the growth medium which, according to the “Ecological Plaque Hypothesis” (Marsh, 2003), is likely to give the acid-tolerant *S. mutans* a competitive advantage through its ability to produce lactic acid from sucrose fermentation. Furthermore, the microbial homeostasis within oral biofilms is suggested to shift when oral environmental changes, such as the ingestion of fermentable sugars, and the creation of low pH environments where acid tolerant/generating organisms thrive and promote de-mineralization. Given consistent sucrose intake, these changes can compound and result in an oral biofilm comprised of predominantly cariogenic pathogens such as *S. mutans*. Our results suggest that established *S. mutans*-free biofilms, both naturally occurring or through STAMP treatment, are able to reduce the competitive advantage of *S. mutans* even in the presence of high sugar content, thus preventing the shift in the biofilm composition toward cariogenesis.

In addition to demonstrating that prior establishment of an *S. mutans*-free biofilm provides considerable protection against subsequent infection with this oral pathogen, we were able to significantly reduce *S. mutans* in the inocula with short 5 minutes exposure, a duration short enough for the application of most oral care products. More importantly, this reduction in *S. mutans* load achieved *via* STAMP treatment resulted in biofilms with considerable protective effects against further *S. mutans* colonization. Taken together, these results suggest that STAMP technology is a potentially useful method for removing *S. mutans*

from saliva, thus establishing an oral flora that can help prevent or reduce further infection of *S. mutans*.

STAMP technology is by no means limited to modulating oral biofilms. It could potentially extend to other simple or complex microbial communities where a dynamic shift in microbial diversity is required to promote a healthy state for the host. Studies have shown that chronic conditions such as Crohn's disease and ulcerative colitis are associated with shifts in the homeostasis of the flora, and it is reasonable to speculate that inter-species interaction plays a role in determining the disease or non-disease state of the complex community within the gut as well. STAMP technology could be used to remove species that have high occurrence rates in patients suffering these diseases, such as *Bacteroides vulgatus* (Mangin et al., 2004), thus helping to reestablish a balanced flora where the specific niche of *B. vulgatus* is filled by other, more benign species. Further studies are underway to examine these potential applications.

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